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ScienceDirect
Journal of Invertebrate Pathology 99 (2008) 74–81
Journal of INVERTEBRATE PATHOLOGY
www.elsevier.com/locate/yjipa

Expressed sequence tags from the red imported fire ant, *Solenopsis invicta*: Annotation and utilization for discovery of viruses

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Received 9 January 2008; accepted 25 January 2008
Available online 1 February 2008

Abstract

An expression library was created and 2304 clones sequenced from a monogyne colony of *Solenopsis invicta*. The primary intention of the project was to utilize homologous gene identification to facilitate discovery of viruses infecting this ant pest that could potentially be used in pest management. Additional genes were identified from the ant host and associated pathogens that serve as an important resource for studying these organisms. After assembly and removal of mitochondrial and poor quality sequences, 1054 unique sequences were yielded and deposited into the GenBank database under Accession Nos. EH412746 through EH413799. At least nine expressed sequence tags (ESTs) were identified as possessing microsatellite motifs and 15 ESTs exhibited significant homology with microsporidian genes. These sequences most likely originated from *Thelohania solenopsae*, a well-characterized microsporidian that infects *S. invicta*. Six ESTs exhibited significant homology with single-stranded RNA viruses (3B4, 3F6, 11F1, 12G12, 14D5, and 24C10). Subsequent analysis of these putative viral ESTs revealed that 3B4 was most likely a ribosomal gene of *S. invicta*, 11F1 was a single-stranded RNA (ssRNA) virus contaminant introduced into the colony from the cricket food source, 12G12 appeared to be a plant-infecting tenuivirus also introduced into the colony as a field contaminant, and 3F6, 14D5, and 24C10 were all from a unique ssRNA virus found to infect *S. invicta*. The sequencing project illustrates the utility of this method for discovery of viruses and pathogens that may otherwise go undiscovered.
Published by Elsevier Inc.

Keywords: *Solenopsis invicta*; Expressed sequence tag; Expression library; Dicistroviridae; Microsporidia

1. Introduction

The black imported fire ant, *Solenopsis richteri*, and red imported fire ant, *Solenopsis invicta*, were introduced into the United States around 1918 (Creighton, 1950) and between 1933 and 1945 (Lennartz, 1973), into Mobile, Alabama, respectively. The black imported fire ant is confined currently to a small area in northern Alabama and Mississippi, and western Tennessee. The more successful red imported fire ant has spread to 128 million hectares from Virginia south to Florida and west to California (Williams et al., 2001) with mound densities as high as 200 per hectare (Macom and Porter, 1996). The red imported fire ant is highly aggressive and considered the dominant arthropod in infested areas (Vinson and Greenberg, 1986). The estimated damage caused by these ants exceeds 1 billion dollars annually in the U.S. (Thompson et al., 1995).

Although chemical insecticides are highly effective at controlling fire ants, they provide only temporary population suppression (Williams et al., 2001). Infested areas cleared of fire ants can only be maintained fire ant free with continuous insecticide use; population levels invariably rebound when insecticide treatment ceases (Tschinkel,

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0022-2011/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.jip.2008.01.004

2006). Because fire ants are so ubiquitous within the infested region of the U.S., insecticide control is impractical. It is generally accepted that the most tenable approach to achieve sustainable control of imported fire ants is the establishment of biological and microbial organisms specific to these ant species (Porter et al., 1997; Tschinkel, 2006). Evidence supporting this notion includes several studies comparing *S. invicta* population characteristics in the United States and in its native range, South America, where fire ants are not considered significant pests. In the U.S., fire ant populations are significantly greater (5-fold), found in higher densities (6-fold), possess larger mound volumes (2-fold), and comprise a larger fraction of the ant community (7.5-fold) than in South America (Porter et al., 1992, 1997). Furthermore, over 30 fire ant natural enemies have been identified in South America—nearly all of which are absent among U.S. populations (Jouvenaz et al., 1977, 1981; Jouvenaz, 1983; Wojcik et al., 1987; Porter et al., 1997; Williams et al., 2003). Thus, as Porter et al. (1997) suggest, *S. invicta* and *S. richteri* likely escaped from their natural enemies in South America when they were introduced into the U.S. Therefore, increasing the number and efficacy of fire ant-specific microbial and biological agents will, with time, lead to self-propagating, sustainable fire ant control.

Expressed sequence tags (ESTs) have been shown to be an effective method of pathogen gene discovery in insects (Hunter et al., 2006; Hunnicutt et al., 2006). An expression library was created and partially sequenced from a fire ant colony with the intention of virus discovery through homologous gene identification A secondary objective, and added benefit of the study, was the identification of genes from *S. invicta* and other associated pathogens providing a resource for further gene characterization.

2. Methods

2.1. Ants

A single monogyne colony of *S. invicta* was used as the source of mRNA for expression library construction. All developmental stages were included in the mRNA preparation, including eggs, larvae, pupae, workers, male and female alates, sexual larvae and pupae, and the queen. The colony was known to be infected with the microsporidian, *Thelohania solenopsae*.

2.2. mRNA extraction and purification

RNA was extracted according to the method of Salzman et al. (1999). Briefly, the entire ant colony (22.5 g) was homogenized in 150 ml of a homogenization solution (4 M guanidinium isothiocyanate, 0.025 M sodium citrate, 0.5% sodium lauroyl sarcosine, 1.5 ml of 2-mercaptoethanol, and 1.5 g of PVP-40) with a baked pestle and mortar. The homogenate was placed into an RNase-free flask and stirred with a stir bar for 10 min. Acid phenol, pH 4.3, (75 ml) was added to the homogenate and allowed to stir for 10 min. Chloroform: isoamyl alcohol, pH 8.0, (24:1, 75 ml), was added to the homogenate and allowed to stir for another 10 min. Aliquots of the homogenate (~35 ml) were added to eight 40 ml Oakridge tubes and centrifuged for 10 min at 12,000*g*. The aqueous layer was transferred to 40 ml centrifuge tubes. Sodium acetate (2 M) was added at a 10% rate and the sample tubes were inverted several times to ensure distribution of the sodium acetate. One volume of isopropanol (at room temperature) was added and the sample was mixed by inversion. RNA was allowed to precipitate overnight at −20 °C. The samples were centrifuged for 20 min at 13,000*g* (4 °C). The pellet was washed with ice-cold 70% ethanol and centrifuged for 10 min at 12,000*g* (4 °C). The ethanol was discarded and the pellets allowed to dry for 15 min at 37 °C and re-suspended in 1.5 ml of DEPC-treated water. The samples were pooled and one volume of lithium chloride added. After incubation overnight at 4 °C, the sample was centrifuged for 20 min at 12,000*g* (4 °C). The pellet was washed with 70% ethanol, dried at 37 °C and re-suspended in 2 ml of DEPC-treated water. The purified total RNA was treated with DNase according to the manufacturer's directions (Invitrogen, Carlsbad, CA). The DNase-treated total RNA samples were pooled and further purified with the MicroPoly(A) Pure RNA extraction protocol following the manufacturer's directions (Ambion, Austin, TX). Purified mRNA was dried and suspended in DEPC-treated water.

2.3. Library construction

A complementary DNA (cDNA) expression library was constructed using the ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Briefly, first strand cDNA was synthesized from polyadenylated mRNA with 5-methyl dCTP and an oligonucleotide primer with an 18-base poly(dT) region appended with an XhoI restriction site. The RNA/DNA hybrid was digested with RNase H and second strand cDNA synthesis was conducted with DNA polymerase I and unmodified dNTPs. EcoRI adaptors were ligated onto the hemimethylated, double-stranded cDNA which was subsequently digested with XhoI producing unidirectional cDNA comprised of an EcoRI restriction site at the 5′ terminus and an XhoI restriction site at the 3′ terminus (relative to the transcript). cDNA was ligated into the Uni-ZAP XR vector and packaged into phage with the Gigapack III Gold packaging kit (Stratagene). Mass excision of the amplified library was carried out using the Ex-Assist (Stratagene) helper phage. Bacterial clones containing the excised pBluescript SK(+) phagemid were recovered by random selection. The bacterial stocks are archived at the USDA-ARS, Center for Medical, Agricultural and Veterinary Entomology, Gainesville, FL, and the Horticulture Research Laboratory, Fort Pierce, FL.

2.4. Sequencing

Insert-positive pBluescript SK(+) phagemids were grown overnight in 96 deep-well plates in LB broth supplemented with ampicillin (100 μg/ml). DNA was extracted with the Qiagen 9600 liquid handling robot and the QIAprep 96 Turbo Miniprep kit according to the manufacturer's directions (QIAGEN Inc., Valencia, CA). Sequencing reactions were completed with the BigDye Terminator v2.0 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Reactions were prepared in 96-well format using the Biomek 2000 liquid handling robot (Beckman Coulter, Inc., Fullerton, CA). Sequencing reaction products were precipitated with isopropanol, re-suspended in sterile water and loaded onto an ABI 3700 DNA Analyzer (Applied Biosystems, Foster City, CA). Inserts were sequenced directionally from the 5′ end of the corresponding transcript (EcoRI restriction site).

2.5. Bioinformatics analysis

Base calling was completed with TraceTuner (Paracel, Pasadena, CA). Low quality bases (quality scores of less than 20) were trimmed from the sequence ends. Quality trimming, vector trimming and sequence alignments were accomplished with the Sequencher software (Gene Codes, Ann Arbor, MI). Sequences comprised of greater than 2% ambiguous bases were not used. Contiguous fragments were first assembled using a minimum overlap of 50 bp and 95% identity. Putative assignment of sequence identity (and EST annotation) was determined by blastx (Altschul et al., 1997) searches of the National Center for Biotechnology Information (NCBI) databases by comparing nucleic acid and protein sequences. Expressed sequence tags (ESTs) were deposited in GenBank (dbEST). ESTs of mitochondrial origin were excluded from the database (Boore, 1999). Sequences with an *E*-score of less than 10^{-5} were considered homologous (Hall, 2004).

Assignment of molecular function of fire ant ESTs exhibiting significant homology was accomplished using the AmiGO tool [Gene Ontology website: http://www.geneontology.org/ (Lomax, 2005)]. AmiGO filters were set as follows: Species—All; Datasource—All; Evidence code—All curator approved; Ontology filter—molecular function.

2.6. ESTs with viral homology

ESTs with significant homology to viral genes were investigated further to confirm their identity. Oligonucleotide primers were designed to each viral EST homolog and additional ant colonies from the field were evaluated by RT-PCR for the presence of the transcript. In some cases, rapid amplification of cDNA ends (RACE) reactions were conducted to acquire additional sequence in an attempt to determine the gene's origin (whether of ant or viral origin).

3. Results and discussion

After vector trimming, ESTs comprised of less than 100 nucleotides or greater than 2% ambiguous nucleotides were excluded from the database set and subsequent analysis. Of the 2304 clones sequenced from the non-normalized fire ant expression library, 1977 quality reads (or 85.8%) were yielded (Table 1). Because multiple copies of the same transcript could be represented, the remaining sequences were assembled into contiguous fragments (contigs). After assembly, 1076 (46.7%) unique sequences (contigs and singlets) were produced representing just under one half of the total clones sequenced. It is most likely that this number is an overestimate of the true number of genes because non-overlapping ESTs representing the same transcript are undoubtedly present. Furthermore, sequencing errors and assembly parameters may have precluded successful assembly of homologous ESTs. The average EST size after assembly was 680 nucleotides.

Although library normalization reduces redundancy and enhances identification of rarer transcripts, sequencing of non-normalized libraries may provide insight into transcript prevalence. For example, in the fire ant EST library, the number of ESTs comprising the contigs ranged from 1 (singlets) to 93 (contig number 2: EH413599)—which represents 8.6% of all the unique sequences after assembly. Similarly, contig 3 (7.3%, EH413565) and contig 13 (4.5%, EH413597) were highly represented. Together, the ESTs comprising these 3 contigs represent 20% of the total number of sequences after assembly. Interestingly, when these contigs were analyzed, no significant matches were identified in GenBank. Thus, they may represent genes unique to *S. invicta*.

Of 1076 unique sequences, 600 (55.8%) exhibited significant homology after blastx (Altschul et al., 1997) analysis with sequences in the GenBank database; 420 (39%) were able to be annotated with a putative function. The remaining sequences either exhibited no significant homology (476 or 44.2%) or exhibited significant homology with unknown function (180 or 16.7%). Therefore, up to 700 (55.7%) of the assembled ESTs potentially represent new genes specific to *S. invicta* or are genes of unknown function. After exclusion of the assembled sequences of apparent mitochondrial and viral origin, 1054 sequences were deposited into the GenBank EST database (dbEST) under the Accession Nos. EH412746 through EH413799.

ESTs exhibiting significant homology after blastx analysis were categorized according to molecular function as assigned by the gene ontology database tool, AmiGO (Fig. 1). The vast majority of these ESTs (~75%) were classified as having "binding" and "catalytic" molecular functions, which is a typical representation (Sabater-Muno et al., 2006). Not surprising, when the fire ant ESTs exhibiting significant *E*-scores were categorized by phylum and

Table 1
Summary statistics for the fire ant EST library

Category	Total number	Proportion[a]
Clones sequenced	*2304*	100
Quality reads	1977	85.8
ESTs submitted to GenBank database (dbEST)	1054	45.7
Sequences of mitochondrial origin	104	4.5
Sequences exhibiting significant homology to microsporidia	15	0.7
Sequences exhibiting significant homology to viruses	6	0.3
Unique consensus sequences after assembly	1076	46.7
Ribosomal (proteins)	159	6.9
Unique consensus sequences after assembly	*1076*	100
Average size of assembled sequences (nucleotides)	680	NA
Singletons	862	80.1
Contigs	214	19.9
Singletons and contigs exhibiting significant homology	600	55.8
Singletons and contigs exhibiting significant homology with unknown function	180	16.7
Singletons and contigs exhibiting no significant homology	476	44.2
Singletons and contigs exhibiting significant homology with known function	420	39.0
Most abundant sequences:		
Contig 2 (EH413599)	93	8.6
Contig 3 (EH413565)	79	7.3
Contig 13 (EH413597)	48	4.5

[a] Total number from category divided by the italicized number at the top of the column.

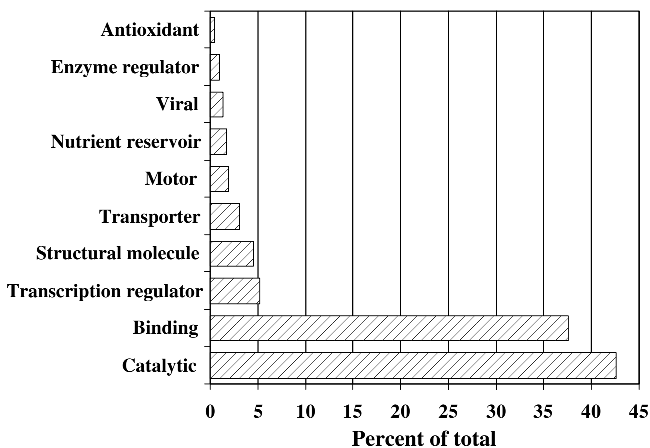

Fig. 1. Assignment of molecular function of fire ant ESTs exhibiting significant homology (excluding ESTs with significant *E*-scores but unknown function) using the AmiGO tool (Gene Ontology website: (http://www.geneontology.org/). Filters were set as follows: Species—All; Datasource—All; Evidence code—All curator approved; Ontology filter—molecular function. Percentages were calculated based on a total of 420 fire ant ESTs.

kingdom (based on the top blast hit for each EST), the majority (64.1%) were homologous to arthropods (Fig 2a) followed by Chordata (7.9%) and Microspora (1.9%). When those ESTs from the Arthropoda group were further categorized by Order, the majority exhibited homology with Hymenoptera (82.8%), Diptera (8.1%) and Coleoptera (4.5%). *Apis mellifera* comprised 90.7% of the hymenopteran homologues. When the fire ant ESTs were compared with those published by Wang et al. (2007), also from fire ant, 60.5% of our sequences exhibited significant matches (*E*-scores less than 10^{-5}).

EST sequences can be a useful source of microsatellite sequences for use as markers for genetic mapping and quantitative genetic analyses (Tagu et al., 2004; Sabater-Muno et al., 2006). At least 9 fire ant ESTs were identified as possessing potential microsatellite motifs (Table 2); eight were dinucleotide and one trinucleotide. Three of the microsatellite repeats were found within putative ORFs (4B11, 21F1, 3E12). The remaining repeats were most likely within the 3′ untranslated region (UTR) because these sequences did not yield significantly large or discernable ORFs (1A8, 2H6, 4B12, 12B9, 16D3, 20D4).

The fire ant colony used for the expression library construction was known to be infected with the microsporidian, *T. solenopsae*. Therefore, it is not surprising that a number of microsporidian-specific ESTs (most likely of *T. solenopsae* origin) were identified by blastx analysis (Table 3). Currently, the only microsporidian genome that has been sequenced is *Encephalitozoon cuniculi* (Katinka et al., 2001). Beyond this, the majority of the microsporidian genes in GenBank are ribosomal. As a consequence of the limited number of microsporidian-specific sequences available for comparison, all of the fire ant ESTs of putative microsporidian origin exhibited homology with *E. cuniculi*. Furthermore, the paucity of microsporidian gene sequences suggests that more of the unidentified fire ant ESTs are likely of *T. solenopsae* origin. The mode of transmission and intercolony infection of *T. solenopsae* in *S. invicta* have not been elucidated. The identification of these genes (most likely from *T. solenopsae*) should facilitate molecular-based methods to pursue these problems.

Despite sequencing a limited number of transcripts (2304) and not normalizing the expression library before sequencing, 6 ESTs of putative viral origin were identified

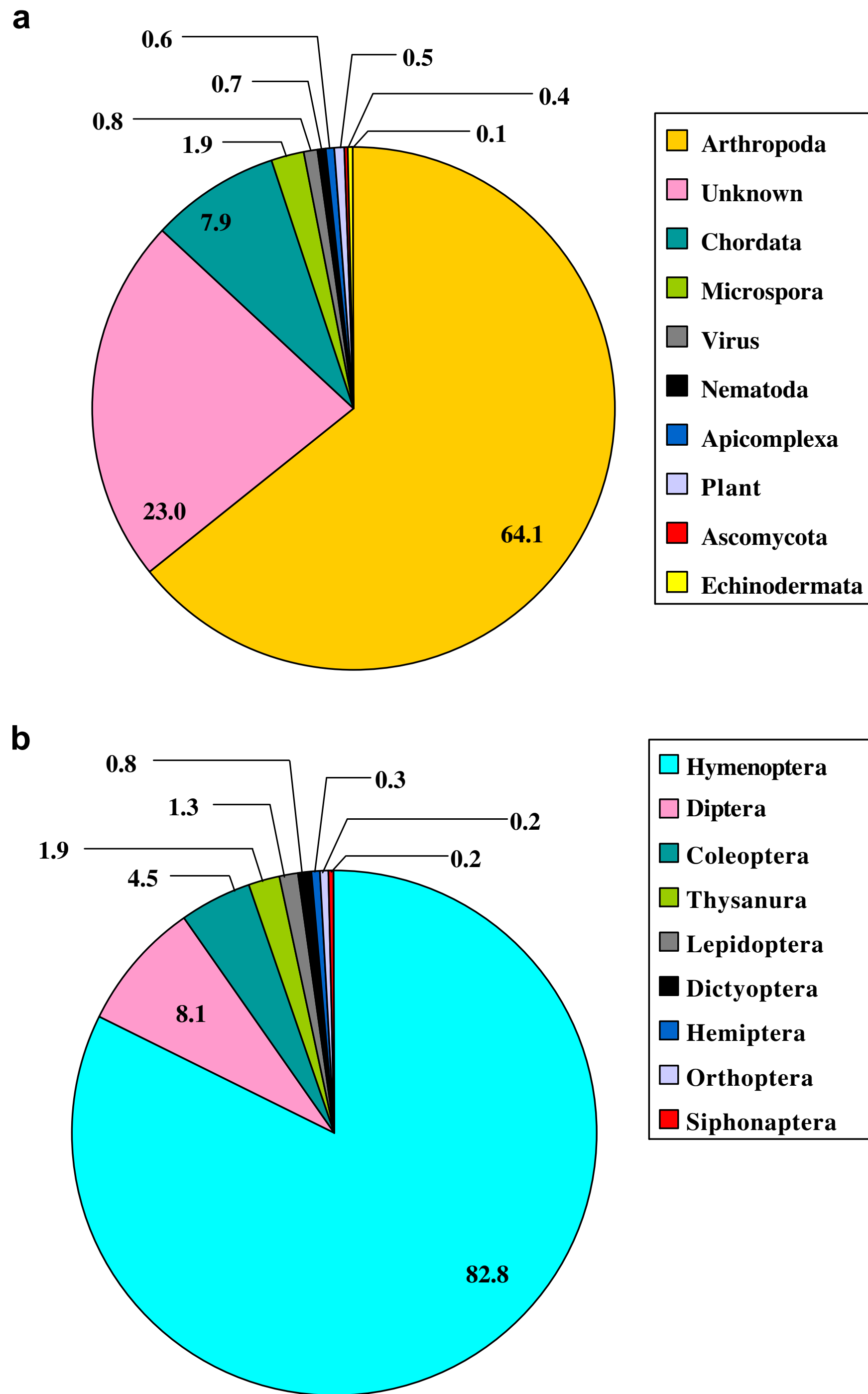

Fig. 2. (a) Distribution of fire ant ESTs by different kingdoms, phyla, virus (b) and insect Orders.

from *S. invicta* (Table 4). All of these sequences exhibited homology with single-stranded RNA (ssRNA) viruses. Each EST with viral homology was examined in an attempt to elucidate its origin, whether viral, ant, or other organism.

The first EST, 3B4 (EF409990), exhibited significant (*E*-score: 3e^{-22}) homology with the Finkel-Biskis-Reilly murine sarcoma virus. Examination of field ($n = 35$) and laboratory-reared ($n = 14$) *S. invicta* colonies by RT-PCR with oligonucleotide primers designed to fire ant clone 3B4 produced an amplicon of anticipated size in 100% of these samples. A RACE reaction was conducted to acquire the 3′ end of this transcript. Subsequent blastx analysis of an ORF from the assembled sequence revealed significant homology to a Braconidae S30 ribosomal protein (*E*-score 2e^{-42}). Because the gene amplified from all *S. invicta* RNA

Table 2
Fire ant ESTs exhibiting microsatellite motifs

Fire ant clone(Accession No.)	Nucleotide		Blast	
	Repeat	Length	Homology	*E*-score
1A8 (DQ987897)	AC	60	No significant homology	>1
2H6 (DQ987898)	TA	26	No significant homology	>1
4B11 (EH412850)	GGC	12	ATPase	$5e^{-61}$
4B12 (DQ987900)	CA	46	No significant homology	>1
12B9 (EH413123)	AT	44	No significant homology	0.6
16D3 (EH413269)	AC	36	No significant homology	>1
20D4 (EH413424)	GT	42	No significant homology	>1
21F1 (EH413471)	AT	30	Unknown function	$4e^{-21}$
3E12 (DQ987899)	CA	54	Septin	$3e^{-6}$

Table 3
Fire ant ESTs exhibiting significant homology with the microsporidian *Encephalitazoon cuniculi*

Fire ant EST clone (Accession No.)	Blast	
	Homology	*E*-score
4C7 (EH412860)	Ribonucleoprotein	$2e^{-30}$
4G5 (EH412876)	Periodic tryptophan protein	$1e^{-8}$
5G10 (EH412915)	DNA lyase/endonuclease 4	$7e^{-59}$
9B5 (EH413005)	ABC transporter-like protein	$4e^{-25}$
9F8 (EH412986)	Chromosome segregation protein	$2e^{-10}$
10G6 (EH413075)	14-3-3 protein	$3e^{-56}$
11B1 (EH413084)	Integral membrane protein	$3e^{-37}$
12A11 (EH413115)	Proteasome 26S ATPase subunit 3	$3e^{-97}$
16C5 (EH413268)	Eukaryotic translation initiation factor 3	$3e^{-32}$
16H5 (EH413286)	26S protease regulatory subunit 6	$1e^{-75}$
18B1 (EH413334)	Ethylene-responsive RNA helicase	$5e^{-49}$
18F4 (EH413350)	Unknown function	$7e^{-57}$
20B7 (EH413403)	RAS-related protein RAB5	$1e^{-47}$
21F6 (EH413479)	Heat shock protein 70	$4e^{-44}$
21F8 (EH413475)	ARF guanine nucleotide exchange factor	$2e^{-9}$

Table 4
Solenopsis invicta ESTs exhibiting significant homology to viruses after blast analysis

Clone (Accession No.)	Blast match	*E*-score
3B4 (EF409990)	Finkel-Biskis-Reilly murine sarcoma virus	$3e^{-22}$
3F6 (AY634314)	Capsid protein, acute bee paralysis virus	$1e^{-17}$
11F1 (DQ112164)	Capsid polyprotein, *Drosophila* C virus	$4e^{-16}$
12G12 (EF409991)	Non-capsid protein, *Urochloa hoja blanca* virus	$5e^{-12}$
14D5 (AY634314)	Capsid protein, acute bee paralysis virus	$1e^{-26}$
24C10 (AY634314)	Capsid protein, acute bee paralysis virus	$2e^{-13}$

samples gathered and the very significant blast *E*-score, it was concluded that fire ant EST 3B4 was most likely a gene from *S. invicta*.

The second EST, 11F1, exhibited significant (*E*-score: $4e^{-16}$) homology with the capsid polyprotein of the *Drosophila* C virus. Examination of worker ants collected from field ($n > 50$) *S. invicta* colonies by RT-PCR with oligonucleotide primers designed to fire ant clone 11F1 produced an amplicon of anticipated size only from the purified plasmid of 11F1; none of the ant samples yielded an amplicon. Through a process of elimination, we realized that crickets being fed to the fire ants were the actual source of the suspected virus (Valles and Chen, 2006).

The third EST, 12G12 (EF409991), exhibited significant (*E*-score: $5e^{-12}$) homology with the non-capsid protein of the plant-infecting *Urochloa hoja blanca* tenuivirus. Examination of field-collected ($n = 10$) *S. invicta* colonies by RT-PCR with oligonucleotide primers designed to fire ant clone 12G12 failed to produce an amplicon. No further tests were performed with this EST because tenuiviruses only infect plants and we failed to detect the virus in field samples. These viruses are often spread by insects (DeMiranda

et al., 2001) so we concluded that 12G12 was most likely acquired from the fire ant diet (either plant or animal).

The remaining ESTs, 3F6, 14D5 and 24C10, all exhibited significant ($1e^{-17}$, $1e^{-26}$, $2e^{-13}$, respectively) homology with the acute bee paralysis virus. After assembly of the ESTs, 3F6 and 14D5 produced a contiguous fragment of ~650 nts. From this ~650 nt assembly, 5′ RACE reactions were conducted which ultimately led to the elucidation of the entire genome of the first virus shown to infect the red imported fire ant, *Solenopsis invicta* virus 1 (SINV-1) (Valles et al., 2004, 2007; Valles and Strong 2005).

The *S. invicta* EST project illustrates the utility of this method for discovery of viruses and pathogens that may otherwise go undiscovered. Extensive searches for fire ant pathogens were conducted in the past in the introduced (United States) and native (South America) ranges of *S. invicta*; none of which observed viral infections (Jouvenaz et al., 1977, 1981; Jouvenaz, 1983). Further complicating discovery of pathogens in fire ants by traditional methods is their fastidious nature (Vinson and Greenberg, 1986). Sick or dying individuals are promptly removed from the nest precluding detection (Vinson and Greenberg, 1986). Additionally, the fire ant EST project has identified a number of unique sequences from *T. solenopsae* (Table 3). These will undoubtedly advance the epidemiology of this disease in fire ants. Lastly, the EST project provides 1054 gene sequences from *S. invicta* that are publicly available through the GenBank database. Certainly these will facilitate a number of studies concerned with fire ant biology.

Acknowledgments

We thank Drs. Rod Nagoshi, Julia Pridgeon (USDA-ARS) and Mike Scharf (University of Florida) for critical reviews of the manuscript. The use of trade, firm, or corporation names in this publication are for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the United States Department of Agriculture or the Agricultural Research Service of any product or service to the exclusion of others that may be suitable.

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